

Title: Conversion of Microalgae Lipids to Fatty Acid Methyl Esters (Biodiesel)

Approvals:

Preparer: _____ Rhykka Connelly _____ Date _____ 04Jul13 _____
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Reviewer: _____ Sonia Wallman _____ Date _____ 04Jul13 _____

1. Purpose:

1.1. To convert microalgal lipids to biodiesel and analyze the conversion efficiency by thin layer chromatography

2. Scope:

2.1. Applies to the production of biodiesel from microalgae.

3. Responsibilities:

- 3.1. It is the responsibility of the course instructor/lab assistant to ensure that this SOP is performed as described and to update the procedure when necessary.
- 3.2. It is the responsibility of the students/technicians to follow the SOP as described and to inform the instructor about any deviations or problems that may occur while performing the procedure.

4. References:

- 4.1. microalgae scale-up SOP
4.2. extraction and analysis of microalgal lipids SOP

5. Definitions: biodiesel – fatty acid methyl esters (FAME), triglyceride (TG), free fatty acid (FFA), thin layer chromatography (TLC)

6. Precautions: Microalgae are live and should be treated with 10% bleach prior to safely discarding. Solvents are used to extract lipids; perform solvent extraction steps under a hood, do not inhale. Ether must be stored in a metal cabinet and discarded 60 days after opening. Store prepared KOH solution in a plastic container.

7. Materials:

- 7.1. 10mL of *Chlorella vulgaris* obtained from UTEX #265 or Carolina Biological #152075 (or bio-prospected)
- 7.2. Rapid Lipid Analysis Kit
- 7.3. methanol (Carolina cat# 861281)
- 7.4. KOH solution (Carolina cat# 883485)
- 7.5. H₂SO₄ solution (Carolina cat# 893301)
- 7.6. hexanes (Carolina cat# 867180)
- 7.7. ether (Carolina cat# 861348)
- 7.8. acetic acid (Carolina cat# 841290)
- 7.9. glass developing jar for TLC
- 7.10. glass visualization jar for TLC
- 7.11. iodine crystals (Carolina cat# 868982)
- 7.12. 15ml centrifuge tubes
- 7.13. 65°C water bath
- 7.14. p1000 pipettor
- 7.15. p1000 pipet tips
- 7.16. 10ml serological pipette tips (Carolina cat# 736125)
- 7.17. p10 pipettor (Carolina cat# 214653)
- 7.18. p10 pipette tips (Carolina cat# 214717)
- 7.19. Pasteur pipets
- 7.20. 2 small plastic bottles with caps

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- 7.21. glass vials (Carolina cat# 715064)
- 7.22. stir plate (Carolina cat# 701012)
- 7.23. stir bar (Carolina cat# 701091)
- 7.24. balance (Carolina cat# 702010)
- 7.25. chemical fume hood

8. Procedure:

8.1. In situ Conversion of Microalgae Lipids to Biodiesel and Analysis of Conversion Efficiency via TLC

- 8.1.1.1. Gather the following items and place on a clean lab bench area:
microalgae culture

- 10mL methanol
- 1ml 10N KOH
- 1ml 10N H₂SO₄
- 90ml hexanes
- 20ml ether
- 1ml acetic acid
- Pasteur pipets
- small beaker (25ml)

- 8.1.1.2. Prepare 10N KOH. On a stir plate, dissolve 30g of KOH to 53ml of dH₂O in an Erlenmeyer flask. Store solution in a plastic bottle.
- 8.1.1.3. Prepare 10N H₂SO₄. Add 27.8ml of 18N H₂SO₄ to 22.2ml of dH₂O. (Always add acid – AAA). Store solution in a plastic bottle.
- 8.1.1.4. Warm a water bath to 65°C.
- 8.1.1.5. Perform a dry cell weight analysis as described in the culture scale-up SOP.
- 8.1.1.6. Pre-weigh a glass vial from the kit.
- 8.1.1.7. Using a 15ml centrifuge tube, centrifuge 10 ml of algal culture at 1000 x g for 5 minutes.
- 8.1.1.8. Discard *all* of the supernatant.
- 8.1.1.9. Resuspend the pellet in 10 ml of methanol. Mix by tapping on the bench several times.
- 8.1.1.10. Incubate in the 65°C water bath for 10 minutes.
- 8.1.1.11. Add 1ml of 10N KOH to the methanolic algae solution. Mix by inverting several times.
- 8.1.1.12. Vent the cap and incubate in the water bath for ≥ 1h.
- 8.1.1.13. Remove the algae from the 65°C water bath. Cool to room temp in cool water bath.
- 8.1.1.14. Under the hood, using the p1000 pipettor, slowly add 1ml 10N H₂SO₄ to the solution. (Dribble the H₂SO₄ down the side of the tube.)
- 8.1.1.15. Replace the cap and mix by inversion several times.
- 8.1.1.16. Vent the cap and incubate in the 65°C bath for ≥ 1h.
- 8.1.1.17. Remove from water bath.
- 8.1.1.18. Add 3ml of hexanes to the solution. Mix by inverting several times. The fatty acid methyl esters will preferentially enter the hexane phase.

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- 8.1.1.19. Phase partition the algae/FAME mixture by centrifuging the sample at 1000 x g for 3 minutes.
- 8.1.1.20. Using a Pasteur pipet, transfer the top hexane phase to a pre-weighed glass vial.
- 8.1.1.21. Add another 3ml of hexane to the same algae mixture and mix thoroughly.
- 8.1.1.22. Centrifuge at 3000rpm for 3 minutes.
- 8.1.1.23. Transfer the hexane phase to the same the glass vial in step 8.1.1.20.
- 8.1.1.24. Allow the pooled hexane phases to evaporate under the hood overnight or apply gentle heat ($\leq 65^{\circ}\text{C}$) under the hood to accelerate the process.
- 8.1.1.25. Once the hexane has evaporated, reweigh the vial.
- 8.1.1.26. Calculate the percentage biodiesel. (Weight of the biodiesel fraction to the weight of the drycell weight of the culture.)

Weight of the glass vial plus biodiesel	_____
Weight of the glass vial	_____
Gravimetric weight of the biodiesel	_____/10 ml of algae culture
Dry cell weight	_____/10 ml of algae culture

Divide the gravimetric weight by the dry cell weight, then multiply by 100 to yield a percentage of biodiesel.

Thin Layer Chromatography

- 8.1.1.27. With gloves, remove TLC plate from sheath. Place the aluminum side down on the bench. The white silica side should face up and have 3 dots near the bottom of the plate. Use care to not disturb the plate as the silica will chip off and affect the migration of the lipids on the plate.
- 8.1.1.28. Resuspend the biodiesel in 100ul of hexane.
- 8.1.1.29. The first lane of the TLC plate has been pre-spotted with lipid standards. Using a p10 with a gel loading tip, take up 5ul of your resuspended biodiesel into the pipet tip. Apply a small amount of the extract onto the second dot on the plate. Allow the solvent to completely evaporate before adding additional extract to the same spot. Continue until all 5ul of the extract are applied to the same spot.
- 8.1.1.30. Using the same technique, spot 5ul of extracted algae lipids (from the algae lipid extraction SOP) onto the 3rd dot on the plate.
- 8.1.1.31. Mix 80ml hexanes, 20ml ether, and 1ml acetic acid together under the chemical fume hood. This is the mobile phase.
- 8.1.1.32. Add the mobile phase to the glass developing jar. The mixture should cover the bottom of the jar, but not exceed the height of the spots on the TLC plate.
- 8.1.1.33. With gloves, transfer the spotted TLC plate to the jar, leaning the plate against the wall of the jar. Make sure that the liquid does not immerse the spotted samples.
- 8.1.1.34. Place the cover on the jar.
- 8.1.1.35. The solvents will carry the lipids up the plate by capillary action.

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- 8.1.1.36. Stop the development once the solvent front is within 1cm of the top of the plate.
Do not allow the solvent front to reach the top of the plate.
- 8.1.1.37. Take the plate out of the developing jar and allow to air dry in a chemical fume hood.
- 8.1.1.38. Add a approximately 1 g of iodine crystals to the visualization jar.
- 8.1.1.39. Transfer the dried plate to the visualization jar. Yellow bands will appear over time.
- 8.1.1.40. Once the bands are visible, take the plate out of the jar.
- 8.1.1.41. Using a cell phone or digital camera, take a digital picture of the plate. The yellow bands will disappear with time.
- 8.1.1.42. With a pencil, draw lines around the yellow bands. If the bands are fading or are difficult to see, the plate can be re-exposed to the iodine crystals to deepen the color density of the bands.
- 8.1.1.43. Pixel density can be measured using a software program called Image J.
- 8.1.1.44. Compare the triglyceride (TG), free fatty acid (FFA), diglyceride (DG) spots in the whole extract lane to the biodiesel sample lane. TG and DG bands should be absent in the biodiesel lane. Some FFA is likely in the biodiesel lane.

9. History:

Name	Date	Amendment
R. Connelly	2011	Initial Release
R. Connelly T. Phelps S. Wallman	2013	Put into 2013 SOP format